Cicatricial Pemphigoid Antigen Differs from Bullous Pemphigoid Antigen by Its Exclusive Extracellular Localization: A Study by Indirect Immunoelectronmicroscopy

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Several components of the dermal–epidermal junction (DEJ) bear the name of the autoimmune bullous disease in which they are involved. The epidermolysis bullosa acquisita (EBA) antigen, a component of anchoring fibrils, and the bullous pemphigoid (BP) antigen, a component of hemidesmosomes (HD) with a molecular weight of 220–240 kD, have been well characterized. In contrast, there is little data known about the cicatricial pemphigoid (CP) antigen. No differences between CP and BP have been reported when sera of patients were studied by Western immunoblotting. Findings of a study of sera from 8 patients with CP by indirect immunoelectron microscopy (IEM) on normal human skin are reported. Saponin (0.1% 10 mn) was used as a permeabilizing agent of cytomembranes and saponin-treated skin samples were compared to saponin-untreated skin samples. Four sera from patients with BP, one from a patient with EBA, and three from healthy donors served as controls. The CP sera produced a similar staining of DEJ on both saponin-treated and saponin-untreated skin samples: immune deposits were localized over the lamina densa and the lower part of the lamina lucida clearly separated from the cytoplasmic membrane of keratinocytes, in regularly spaced clumps. The BP sera produced an intense staining of DEJ only on saponin-treated skin samples: immune deposits were observed on the cytoplasmic attachment plaque of the HD; on saponin-untreated skin samples, BP sera produced only a faint staining of the extracellular part of HD. Finally, as expected the EBA serum appeared on the lower part of the lamina densa and anchoring fibrils, and no DAB deposits were observed with the serum of healthy donors. This data indicated that CP antigen is different than BP antigen by its exclusive extracellular localization. It may be a component of anchoring filaments. *J Invest Dermatol 97:3–9, 1991*

Several indirect immunoelectron microscopic (IEM) studies clearly demonstrate that within normal skin the bullous pemphigoid (BP) antigen is associated with hemidesmosomes (HD) and distributed in two pools. On one hand, there is a large intracellular pool, closely related to the attachment plaque of HD and associated tonofilaments; on the other, a small extracellular pool, the external part of HD [1–7]. In the skin of patients with BP, only the extracellular pool of the BP antigen is recognized by autoantibodies because large molecules such as immunoglobulins cannot penetrate the cytoplasmic membrane of basal keratinocytes. Consequently, by direct IEM, in vivo bound autoantibodies deposits are seen only in the lamina lucida, usually at its upper portion [2–3, 5–6, 8–11].

In cicatricial pemphigoid (CP), only few IEM studies of in vivo bound autoantibodies have been reported, and in these the immune deposits are seen over the lower part of the lamina lucida and over the lamina densa [10,12,13]. To our knowledge, no study by indirect IEM of the fine localization of CP antigen within normal skin, has been reported.

In the present study, we explored by indirect IEM, a potential intracellular pool of CP antigen, unknown by direct IEM. Saponin was used as a permeabilizing agent. CP sera were tested concurrently with BP sera. This study demonstrates that contrary to BP antigen, CP antigen is not distributed inside the keratinocytes and, consequently, BP and CP antigens can be clearly differentiated by their ultrastructural localization within dermal–epidermal junction (DEJ). Our results suggest that the CP antigen might be a component of anchoring filaments in relation with their insertion on the lamina densa.

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Abbreviations:
BP: bullous pemphigoid
CP: cicatricial pemphigoid
DAB: 3, 3′-diaminobenzidine
DEJ: dermo–epidermal junction
EBA: epidermolysis bullosa acquisita
ELISA: enzyme-linked immunosorbent assay
HD: hemidesmosome
IEM: immunoelectron microscopy
IIF: indirect immunofluorescence

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Table I. Indirect Immunofluorescence and Western Immunoblot Data

Patients[b]	Indirect Immunofluorescence (titer)			Western Immunoblot[a]		
	Rat Esophagus	Normal Human Skin	Split Normal Human Skin	Epidermal Extracts		Dermal Extracts
1	0	1:10	>1:40 (roof)	180	—	—
2	0	ND[c]	ND	—	240	—
3	ND	1:10	1:40 (roof)	180	—	—
4	0	0	1:10 (roof)	180	240	—
5	0	0	0	—	240	—
6	0	0	1:20 (roof)	180	—	—
7	0	1:10	1:50 (roof)	180	—	—
8	0	0	0	—	—	—
9	ND	1:3200	+ (roof)	—	240	—
10	1:2560	1:320	1:320 (roof)	—	240	—
11	1:2560	1:320	1:320 (roof)	—	240	—
12	1:2560	1:80	1:40 (roof)	—	240	—
13	ND	1:320	+ (floor)	—	—	290
14	ND	0	0	—	—	—
15	ND	0	0	—	—	—
16	ND	0	0	—	—	—

[a] Molecular weight in kilodalton (kD).
[b] Patients: 1–8, CP; 9–12, BP; 13, EBA; 14–16, Controls.
[c] ND, not determined.

MATERIALS AND METHODS

Patients Eight patients with CP (patients 1–8) were compared to four patients with BP (patients 9–12). Patients 1 to 6* had a classic CP, based on the predominance of mucosal involvement, chronic course of the disease, and scars on the trunk, head and neck. Patient 7 had a chronic scarring cutaneous localized form of the Brunstig–Perry type and patient 8 a desquamative gingivitis. Patients 9 through 12 had clinical features of typical BP. In addition, one patient with epidermolysis bullosa acquisita (EBA) (patient 13) and three healthy donors (patients 14–16) served as controls. All patients 1 through 13 had, by direct immunofluorescence, linear IgG or C3 deposits along the DEJ.

Indirect Immunofluorescence and Western Immunoblotting Sera were obtained from the patients at the time of diagnosis and stored at −70°C. All sera had been tested both by indirect immunofluorescence (IIF) and Western immunoblotting. IIF was performed on rat esophagus sections and/or normal human skin (Table I), and sodium chloride-split normal human skin as recommended by Gammon [14]. Western immunoblot analysis was performed on both epidermal and dermal extracts as previously reported [13].

Immunoelectron Microscopy Direct IEM was performed using a previously described technique [15]. All the patients were studied except the one with BP and the healthy donors. Indirect IEM was performed as described above.

Normal human skin was obtained from the abdominal area of healthy donors. Six millimeter punch biopsies were performed.They were immediately cut into 0.7-mm-thick slices with a hand microtome [16] and washed in Hanks' medium for 15 min. Then half of the slices (the saponin-treated skin samples) were immersed in Hanks' medium with 0.1% saponin for 10 min, washed with Hanks' solution, and incubated with a patient serum, at a dilution of 1:5 with a CP serum or 1:32 with a BP serum, for 24 h at 4°C under agitation. The other half of the slices (the saponin-untreated skin samples) were incubated with the same sera in the same conditions.

Thereafter, the procedure used was very similar to the procedure reported in direct IEM [15], namely, fixation in 4.5% buffered formaldehyde, incubation with horseradish peroxidase-labeled goat polyclonal antibody, antihuman gamma heavy chain (Pasteur), at a dilution of 1:10 for 24 h, fixation in Karnovsky medium, incubation in Graham and Karnovsky medium, post-fixation in OsO_4, and then embedding in epoxy resin that allowed the cutting of a large number of both semithin and ultrathin sections.

Ultrathin sections were systematically examined with a Philips EM 301 electron microscope at magnifications of both ×4,500 and ×12,500. All the ultrathin sections were examined in a blind study by two observers. The low magnification (×4,500) allowed the study of the staining contrast compared to background on a long stretch of the DEJ. The intensity of this contrast was scored from 0 (absence of staining) to +++ (intense staining with marked contrast). High magnification provided the fine ultrastructural localization of the deposits within the DEJ.

RESULTS

Indirect Immunofluorescence and Western Immunoblotting In all patients' sera, except the serum of patient 8, circulating autoantibodies were detected by IIF or Western immunoblotting. Results are summarized in Table I. By IIF, titers were rather low in CP and high in BP. In both CP and BP, on the saline-treated split skin, all positive sera were found on the roof of the blister. In contrast, the EBA serum was the floor of the blister. By immunoblotting on epidermal extracts, two of the eight CP sera recognized a band of 240 kD, four CP sera a band of 180 kD, and one CP serum the bands of both 240 kD and 180 kD; all four BP sera recognized the major determinant of BP antigen of 240 kD. By immunoblotting on dermal extract, the EBA serum recognized a 290-kD band. In healthy donors, no circulating autoantibodies were detected by either IIF or immunoblotting.

Direct Immunoelectron Microscopy In all of the patients with CP (patients 1–8), the immune deposits were situated both on the lamina lucida and the lamina densa (Fig 1*A*). They were thick and irregular, and produced a discontinuous pattern along the DEJ (Fig 1*B*). In three of the four BP patients who have been studied, immune deposits were strictly localized in the lamina lucida. Finally, in the patient with EBA, the immune deposits were situated below the lamina densa in the anchoring fibril zone.

* Patients' sera 1 to 4 have been previously studied [13].

Indirect Immunoelectron Microscopy During the blind observation under the electron microscope, nothing (including cellular damage or modification of background) indicated whether skin samples had been treated by saponin.

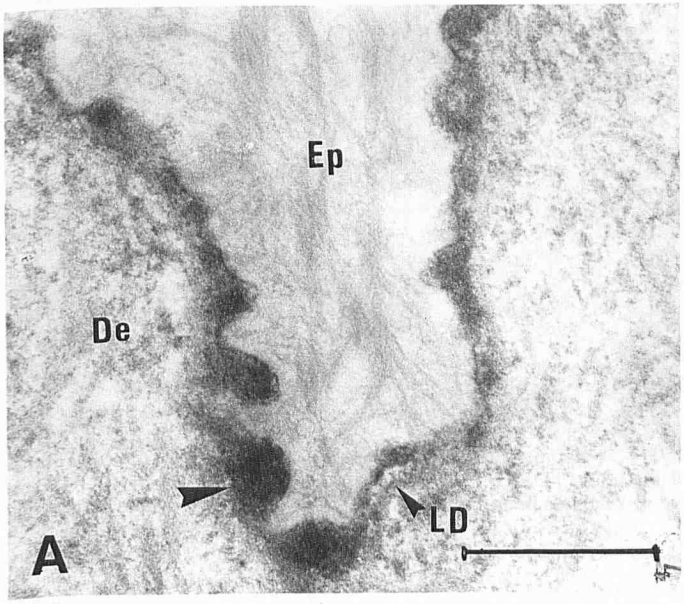
All the CP sera, except the serum of patient 8, produced a similar staining of DEJ with or without prior treatment by saponin (Fig 2) (Table II). At low magnification when the staining was intense (Fig 2*A,C*), a particular "crenelated" pattern was observed. At high magnification (Fig 2*B–D*), the deposits were situated over the lamina densa, in clumps adjacent to the lamina densa, and were regularly spaced in the lamina lucida. An unstained, clear space was always seen between the deposits and the cytoplasmic membrane of the basal keratinocytes. No deposits were seen within the basal keratinocytes in either saponin-treated or in untreated skin samples. With the CP serum from patient 8, in which no circulating antibodies were detectable by either IIF or Western immunoblot, only a faint staining of DEJ was observed on both saponin-treated and -untreated skin samples.

Results with the CP sera were quite different from those observed with BP sera, as staining of DEJ by indirect IEM with all of the four BP sera were not at all similar in saponin-treated and -untreated skin samples (Fig 3) (Table II). After saponin treatment, at low magnification (Fig 3*A*), an intense and discontinuous staining was observed on the epidermal side of the DEJ. At higher magnification (Fig 3*B*), strong deposits were seen over the intracellular part of the HD, which were bulging in the lamina lucida. However, a free lamina lucida space could always be seen between the DAB deposits and the lamina densa. Without saponin treatment at low magnification (Fig 3*C*), the staining on the DEJ was very weak or absent. At high magnification (Fig 3*D*), only the extracellular parts of the HD were slightly stained.

Our results with the CP and the BP sera were also quite different from those observed in EBA; on saponin-treated skin samples, the EBA serum decorated the lower part of the lamina densa and the anchoring fibrils, whereas the lamina lucida and the HD remained unlabeled (Fig 4). Finally, in controls with normal human serum, similar results were observed on both saponin-treated and saponin-untreated skin. Contrast was very weak, without any staining of the lamina densa. High magnification showed that the external part of the HD was outlined. The attachment plaque of the HD was sharply demarcated (Fig 5).

DISCUSSION

CP and BP are two subepidermal bullous diseases characterized by the binding of autoantibodies (IgG) or C3 along the DEJ. Different clinical features in BP and CP, in particular the scarring evolution of cutaneous lesions and the predominance of mucosal involvement in CP [17,18], suggest that differences in the target antigens are recognized by the autoantibodies. To date, target antigens in BP and CP cannot be differentiated by their molecular weight. In BP, using Western immunoblotting of epidermal extracts or immunoprecipitation, most of the patients' sera label a 230-kD protein, the so-called major BP antigen, some sera label a 180-kD one, and very few label a 97- or a 77-kD one [19–22]. The Western immunoblot assay reports on CP in the literature, indicate that most of the patients'



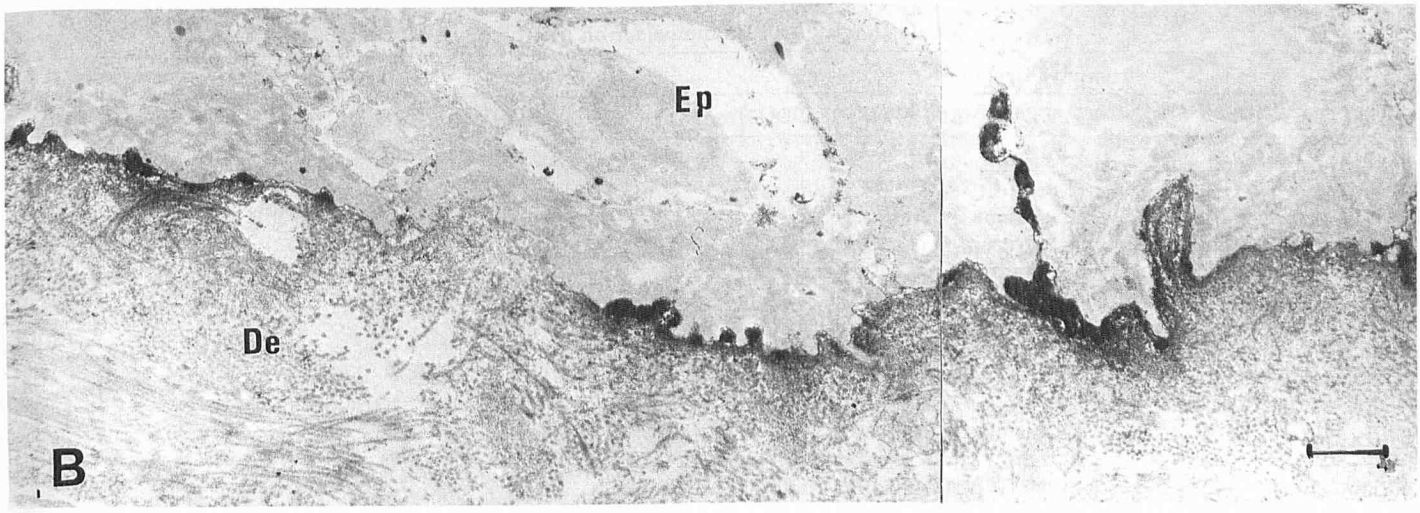


Figure 1. Direct IEM in CP patients. *A,* High magnification (patient 7, scarring cutaneous localized form). Immune deposits on the lamina lucida and the lamina densa (LD). Ep, epidermis; De, dermis. (Magnification ×50,000; *bar,* 0.5 μm.) *B,* Low magnification (patient 5). Thick, irregular, and discontinuous deposits along the DEJ. Ep, epidermis; De, dermis. (Magnification ×10,000; *bar,* 1 μm.)

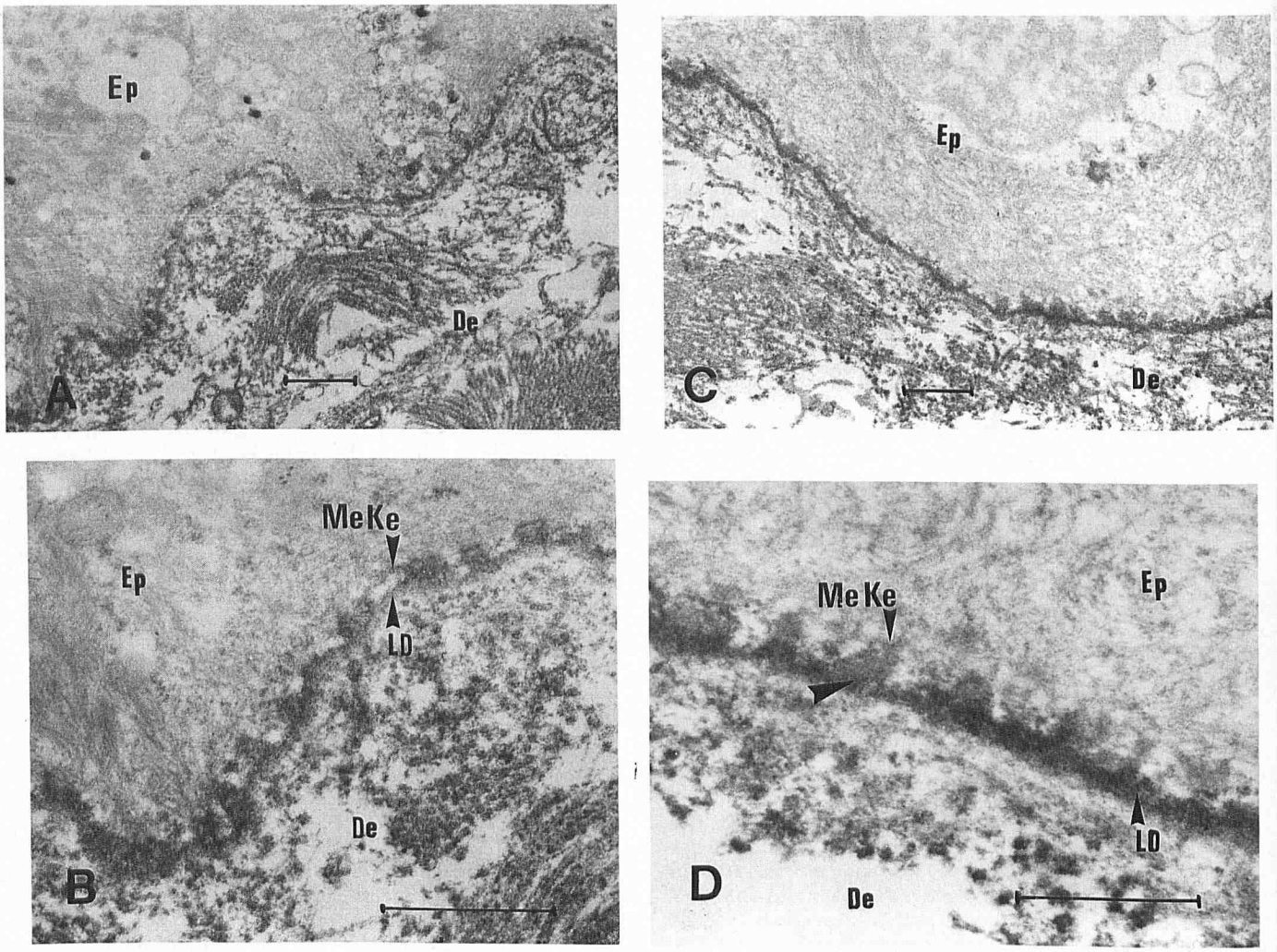


Figure 2. Indirect IEM in CP patients. *A,B,* Saponin-treated skin samples (patient 7); *C,D,* saponin-untreated skin samples (patient 2). *A,C,* Low magnification (×4,500; *bar,* 1 μm); *B,D,* high magnification (×12,500; *bar,* 1 μm). *A,C,* Similar staining of DEJ. *B,D,* Immune deposits on the lamina densa (LD) and on the lower part of the lamina lucida. A clear space is seen between deposits and cytoplasmic membrane of keratinocytes (Me Ke).

Table II. Indirect Immunoelectron Microscopy

	Saponin +[b]							Saponin −[c]						
Patients[a]	×4500	IHD[d]	EHD[d]	Af	LL[d]	LD[d]	AF[d]	×4500	IHD[d]	EHD[d]	Af[d]	*LL*[d]	LD[d]	AF[d]
1	+++	−	+	+	−	+	−	++	−	−	+	−	+++	+
2	++	−	+	+	−	+	−	++	−	−	+	−	+	−
3	++	−	−	+	−	+	−	+++	−	−	+	+	+	−
4	+	−	+/−	+	−	+	−	++	−	+	+	−	+	−
5	+++	−	−	+	−	+	−	++	−	−	+	−	+	−
6	+++	−	+	+	−	+	−	+++	−	−	+	−	+	−
7	+++	−	+	+	−	+	−	+++	−	+	+	−	+	−
8	+	−	−	+	−	−	−	++	−	++	+	−	−	−
9	+++	+++	+	−	−	+/−	−	−	−	+/−	−	−	−	−
10	+++	++	−	−	−	−	−	+	−	++	−	−	−	−
11	+++	++	−	−	−	−	−	+	−	++	−	−	−	−
12	++	++	−	−	−	−	−	+	−	++	−	−	−	−
13	+++	−	−	−	−	−	+				ND			
14	+/−	−	+	−	−	−	−	+	−	+	−	−	−	−
15	−	−	−	−	−	−	−	−	−	−	−	−	−	−
16	+	−	+	−	−	+/−	−				ND			

[a] Patients: 1–8, CP; 9–12, BP; 13, EBA; 14–16, controls.
[b] Saponin-treated skin samples.
[c] Saponin-untreated skin samples.
[d] IHD, internal hemidesmosome; EHD, external hemidesmosome; Af, anchoring filaments; LL, lamina lucida; LD, lamina densa; AF, anchoring fibrils.

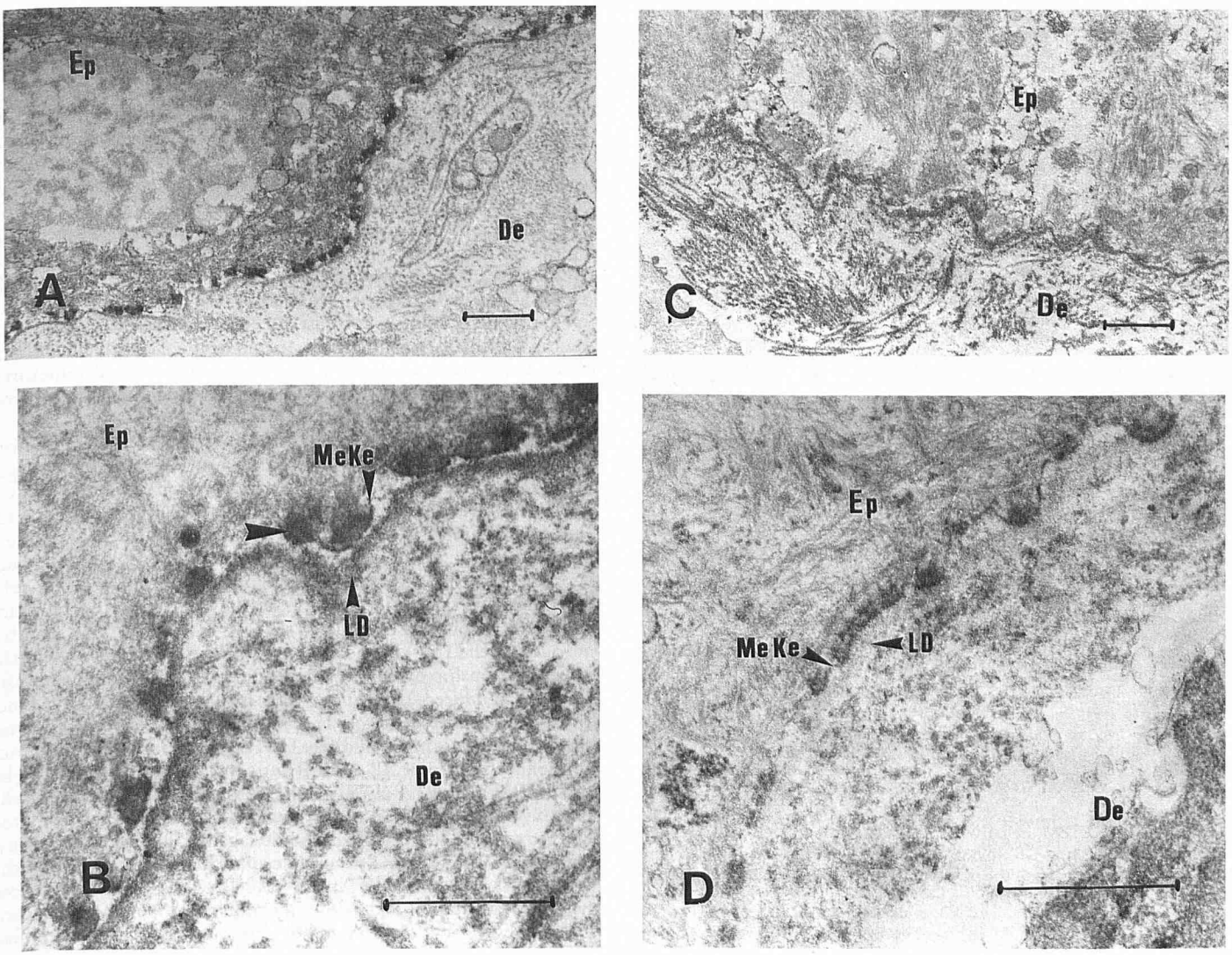


Figure 3. Indirect IME in BP patients. *A,B,* Saponin-treated skin samples (patient 7); *C,D,* saponin-untreated skin samples (patient 2); *A,C,* low magnification (×4,500; *bar,* 1 μm); *B,D,* high magnification (×12,500, *bar,* 1 μm). *A,* Intense staining of DEJ (patient 10). *B,* Immune deposits on the internal part of hemidesmosomes. A clear space is seen between the cytoplasmic membrane of keratinocytes and the lamina densa *(LD)* (patient 9). *C,* Faint staining of DEJ (patient 12). *D,* Absence of immune deposits on the internal part of hemidesmosomes. The attachment plaque is sharply demarcated. Only the external part of the hemidesmosomes is outlined (patient 12).

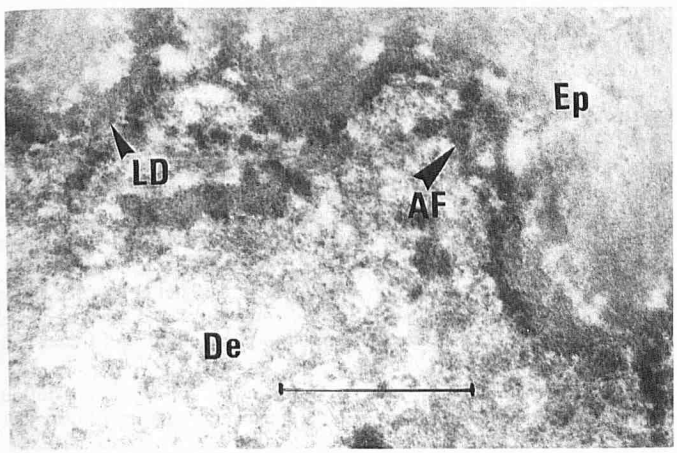


Figure 4. Indirect IEM in EBA (patient 13). The EBA serum decorates the lower part of the lamina densa *(LD)* and anchoring fibrils *(AF)* (magnification ×25,000; *bar,* 1 μm).

sera recognize epidermal proteins similar to BP antigens, of molecular weight 240 or 180 kD [13,22,23]; only two patients, in one study, had circulating autoantibodies that recognized a different protein of 120 kD [23]. In contrast, some reports suggest that CP and BP target antigens could be differentiated by their fine localization with DEJ. First, localizations of autoantibodies bound in vivo in lesional skin, as determinated by direct IEM, are different in BP and CP. In BP, immune deposits are thin, continuous, and situated in the upper part of the lamina lucida, whereas in CP, they are thick, irregular, discontinuous, and situated in the lower part of the lamina lucida and on the lamina densa [12,13]. Second, binding of circulating autoantibodies on saline-treated split normal human skin by indirect immunofluorescence are different; in BP, all the patient sera decorated the roof of the cleavage, whereas in CP both the roof and the floor of the cleavage were labeled [24]. More recently, studies of ultrastructural localization of BP antigen by indirect IEM [1–7] have explained this pattern observed in indirect IF on saline-treated split skin. A pool of BP antigen, the largest one of which is intracellular, situated on the attachment plaque of HD and a second one that is extracellular and closely associated with the keratinocyte

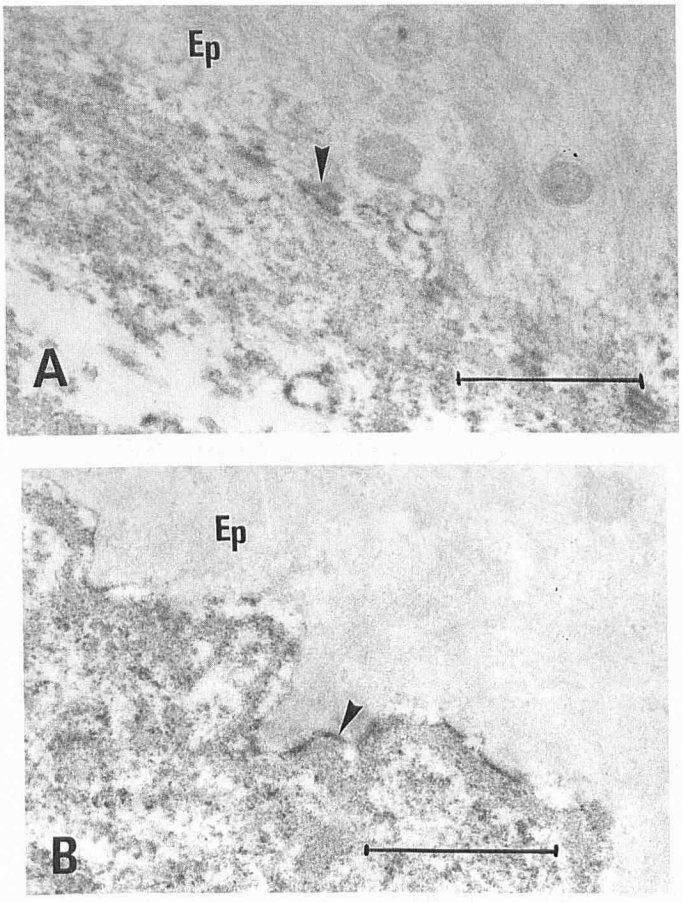


Figure 5. Indirect IEM, negative controls. *A*, Saponin-treated skin; *B*, on saponin-untreated skin. *A,B,* In only one of the normal human sera (patient 14), are the external parts of the hemidesmosomes *(arrow)* outlined (magnification ×12,500, *bar,* 1 μm).

cytoplasmic membrane on the external part of HD. By indirect IEM, no difference in the labeling of HD was observed between affinity-purified antibodies directed against the 230-kD epitope and those directed against the 180-kD one [25]. To our knowledge, no study by indirect IEM of the ultrastructural localization of CP antigen within normal human skin has been reported. In the present study, we compare fine localizations of BP and CP antigens using indirect IEM, to know precisely whether there is an intracellular component in CP, as in BP, that is, undetected by direct IEM. We have tested, comparatively, the sera of eight patients with CP and four with BP. All of these patients had typical clinical features and typical direct IEM. In addition, all the sera have been characterized by immunoblotting on epidermal and dermal extracts.

Different techniques of indirect IEM used to reveal the intracellular pool of BP antigen, have been reported in the literature, namely, post-embedding or pre-embedding techniques. In post-embedding techniques [7], an "on-section" labeling, is performed on cryofixed, cryosubstituted, and in Lowicryl-embedded skin samples. When a pre-embedding IEM technique is used, a permeabilization of the cell membrane is necessary to expose the intracellular antigens [26], otherwise no deposits can be observed in BP [27]. Freezing and thawing, or enzyme digestion, exhibit the intracellular pool, but unfortunately induce a marked alteration of morphology and tissue architecture. We want to emphasize that in our study by indirect IEM, the biopsies were never frozen and saponin (0.1%), a detergent that produces holes in the lipid bilayer of the plasma membrane [2, 26], was used to permeate the cell membrane. Consequently, the morphology of the saponin-treated skin samples remained satisfactory and similar to the saponin-untreated skin samples. Recently, another method used to study the binding of bullous pemphigoid sera to intracellular or extracellular epitopes using viable or permeabilized SCaBER cells as substrate for an enzyme-linked immunosorbent assay (ELISA) has been reported [28]. When the BP sera that recognized the 230-kD antigen by immunoblotting were tested, an increase of specific binding to permeable cells, compared with viable cells indicated the recognition of intracellular epitopes. When the BP sera that recognized the 180-kD antigen by immunoblotting were tested, similar specific binding to both viable and permeabilized cells indicated the recognition of extracellular epitopes. Our results with BP sera, which recognized the 240-kD antigen by immunoblotting, confirm the data in the literature. In saponin-treated skin, the deposits were intracellular on the attachment plaque of HD. In contrast, on saponin-untreated skin only a staining of the extracellular component of the BP antigen could be seen. These extracellular deposits were very faint and sometimes difficult to differentiate from the unspecific staining observed on the control samples.

In contrast, CP sera produced the same staining pattern on both saponin-treated and saponin-untreated skin. This is a decisive argument for an exclusively extracellular localization of the antigen. CP antigen seems to be situated in front of the HD in the lamina lucida and over the upper part of the lamina densa. It could be a component of the anchoring filaments and the biochemical support of the enlargement of the lamina densa facing the HD [29]. In our CP sera, no difference in the localization of the deposits could be made between CP sera that recognized the 240-kD band and CP sera that recognized the 180-kD one. For more precise comprehension of the exact relationship of the CP antigen with HD, it would be helpful to perform indirect IEM studies in hereditary diseases such as junctional epidermolysis bullosa that affect the hemidesmosome complex. In conclusion, this indirect IEM study confirms the difference in the localization of BP and CP antigens, previously suspected by direct IEM studies. A cDNA clone with coding sequences for the carboxy terminal region of BP antigen has been recently isolated and sequenced [30]. Rabbit antibodies against the carboxy terminal epitopes have been recently produced [31]. They bind only to the intracellular part of HD. Similar studies on CP antigen are now necessary to comprehend further the molecular basis and the pathogenesis of the two diseases and determine whether there are two different proteins, or rather the same protein with different epitopes situated on each side of the lamina lucida.

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